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Hedgehog signaling induces arterial endothelial cell formation by repressing venous cell fate

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ABSTRACT

In vertebrate embryos, the dorsal aorta and the posterior cardinal vein form in the trunk to comprise the original circulatory loop. Previous studies implicate Hedgehog (Hh) signaling in the development of the dorsal aorta. However, the mechanism controlling specification of artery versus vein remains unclear. Here, we investigated the cell-autonomous mechanism of Hh signaling in angioblasts (endothelial progenitor cells) during arterial–venous specification utilizing zebrafish mutations in Smoothened (Smo), a G protein-coupled receptor essential for Hh signaling. *smo* mutants exhibit an absence of the dorsal aorta accompanied by a reciprocal expansion of the posterior cardinal vein. The increased number of venous cells is equivalent to the loss of arterial cells in embryos with loss of Smo function. Activation of Hh signaling expands the arterial cell population at the expense of venous cell fate. Time-lapse imaging reveals two sequential waves of migrating progenitor cells that contribute to the dorsal aorta and the posterior cardinal vein, respectively. Angioblasts deficient in Hh signaling fail to contribute to the arterial wave; instead, they all migrate medially as a single population to form the venous wave. Cell transplantation analyses demonstrate that Smo plays a cell-autonomous role in specifying angioblasts to become arterial cells, and Hh signaling-depleted angioblasts differentiate into venous cells instead. Collectively, these studies suggest that arterial endothelial cells are specified and formed via repressing venous cell fate at the lateral plate mesoderm by Hh signaling during vasculogenesis.

Introduction

In zebrafish embryos, progenitor cells of the dorsal aorta and the posterior cardinal vein migrate sequentially to the midline and coalesce to form the vascular cord. Subsequently, this endothelial cord undergoes differentiation and morphogenesis to form the dorsal aorta and the posterior cardinal vein (Jin et al., 2005; Zhong et al., 2001; Herbert et al., 2009). Despite recent progress in dissecting the regulation of arterial and venous endothelial cell differentiation (Lawson, 2002; Swift, 2009; Zhong, 2005), the mechanisms that control specification and formation of arterial and venous endothelial cells are not well understood.

Hedgehog (Hh) signaling plays important roles in specification and formation of a variety of cell types and organs during development (Ingham and McMahon, 2001). It has been shown to be involved in neovascularization and angiogenesis. In mouse, loss of *sonic hedgehog* (*shh*) causes lack of proper vascularization of the developing lungs (Rowitch et al., 1999), whereas *shh* overexpression in the dorsal neural tube results in hypervascularization (Pepicelli et al., 1998). In the developing heart, a wave of Hh activation is required for coronary vascular development (Lavine et al., 2008, 2006). *shh* expression upregulates *vegf* and *angiopoietins* to enhance myocardial neovascularization in ischemic hearts (Kusano et al., 2005; Pola et al., 2001). Inactivation of the *smoothened* (*smo*) gene, encoding the co-receptor for Shh, Indian hedgehog (Ihh) and Tiggy-winkle hedgehog (Twhh), causes severe angiogenesis defects in the yolk sac of mouse embryos (Byrd et al., 2002).

Hh signaling has also been implicated in formation and differentiation of the dorsal aorta in zebrafish. Zebrafish *smoothened* mutants lack the dorsal aorta resulting in absence of all arterial gene expression (Gering and Patient, 2005) . Mutations in *sonic-you* (*syu*) and *you-too* (*yot*), which encode zebrafish *shh* ortholog and its downstream effector *gli2*, respectively, display defects in arterial endothelial differentiation (Brown et al., 2000; Chen et al., 1996; Lawson et al.,

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2002). These data suggest that Hh signaling is required for formation of the dorsal aorta at early stages as well as for arterial endothelial differentiation during later developmental stages. Hh signaling has been implicated to regulate arterial endothelial differentiation via modulating Vegf (Lawson et al., 2002). Studies in avian and murine embryos, however, indicate that Hh signaling acts independently of Vegf to mediate vascular tubulogenesis and arterial assembly (Vokes et al., 2004). Although these studies reveal early roles for Hh signaling in artery development, it remains unclear when, where or how Hh signaling controls formation of the dorsal aorta. In addition, little is known about whether the absence of the dorsal aorta has any effect on development of the cardinal vein in Hh-deficient embryos.

In this study, we have analyzed early roles of Hh signaling in arterial–venous specification and identified the cell-autonomous requirement for reception of Hh signaling by endothelial progenitor cells to form the dorsal aorta. In Hh signaling-deficient embryos, we observed a reciprocal expansion of the cardinal vein that is accompanied by an absence of the dorsal aorta, whereas activated Hh signaling by Smo agonist causes arterial cell expansion that is proportional to reduction of venous cells. These data suggest that arterial endothelial cells develop at the expense of venous cell fate. In vivo time-lapse imaging has revealed two waves of migrating endothelial progenitor cells. The first wave contributes, mainly, to the formation of the dorsal aorta, and the second wave gives rise to the posterior cardinal vein. Angioblasts deficient in Hh signaling all migrate in the second (venous) wave to form an expanded cardinal vein. Our cell transplantation analyses demonstrated that reception of Hh signaling by angioblasts is required for them to adopt an arterial cell fate and contribute to formation of the dorsal aorta, whereas angioblasts with impaired Hh signaling differentiate into venous cells instead.

Methods and materials

Zebrafish maintenance and staging

Embryos were produced by pairwise matings and raised at 28.5 °C, then staged according to hours post fertilization (hpf) and days post fertilization (dpf) (Kimmel et al., 1995). *Smo*^{hi640} null allele (Chen et al., 2001) and transgenic fish included *Tg(flk:EGFP)* (Jin et al., 2005), *Tg(fli:EGFP-nuc)*(Roman et al., 2002) and *Tg(flk:DsRed)* (Huang et al., 2005) were used in our studies.

Cyclopamine and purmorphamine treatment

Embryos were incubated in Embryo Medium (EM) containing 50 μM and 100 μM of cyclopamine or purmorphamine respectively. Treatments were carried out at 50% epiboly unless stated otherwise.

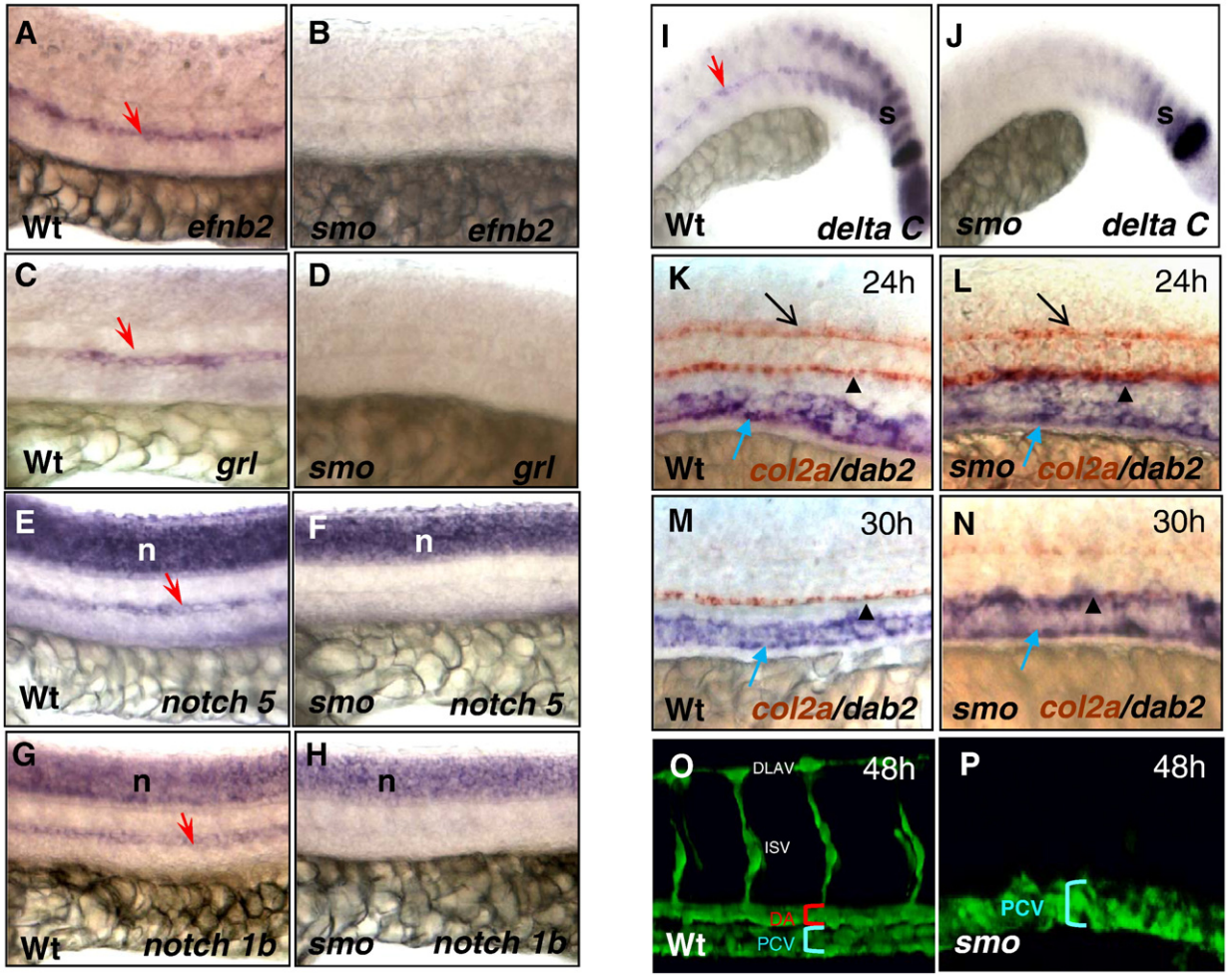

Fig. 1. *smo* mutants display expansion of the posterior cardinal vein and absence of the dorsal aorta. (A–J) Lateral views displaying absent expression of *efnb2* (A, B), *grl* (C, D), *notch5* (E, F), *notch1b* (G, H) and *deltaC* (I, J) in *smo* mutants, compared to wild-type embryos. (K–N) Double in situ hybridization exhibiting the expansion of *dab2* venous domain and *col2a* expression in the neural tube and the hypocord in *smo* mutants (L, N) compared to wild-type embryos (K, M). (O, P) Confocal microscopy depicting the dorsal aorta and the posterior cardinal vein in wild-type embryos [*Tg(flk:EGFP)*] (O); and absence of the dorsal aorta and expansion of the posterior cardinal veins in *smo* mutants [*smo*^{hi640}/*smo*^{hi640}; *Tg(flk:EGFP)*] (P). Red arrow: dorsal aorta. Blue arrow: cardinal vein. Black arrow: floor plate. Black arrowhead: hypocord. s: somite. n: neural tube. DA: dorsal aorta. PCV: posterior cardinal vein. DLAV: dorsal longitudinal anastomotic vessel.